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Title Polyphenolic extract attenuates fatty acid-induced steatosis and oxidative stress in hepatic and endothelial cells

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2 **hepatic and endothelial cells**

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ABSTRACT

Purpose

Polyphenols (PP) of virgin olive oil exert several biochemical and pharmacological beneficial effects. Some dietary PP seem to prevent/improve obesity and metabolic-related disorders such as non-alcoholic fatty liver disease (NAFLD). We investigated the possible effects of PP extracted from olive pomace (PEOP) and of the main single polyphenols present in the extract (tyrosol, apigenin, oleuropein, p-coumaric and caffeic acid) in protecting hepatocytes and endothelial cells against triglyceride accumulation and oxidative stress.

Methods

Rat hepatoma and human endothelial cells were exposed to a mixture of oleate/palmitate to mimic the condition of NAFLD and atherosclerosis, respectively. Then, cells were incubated for 24 h in the absence or in the presence of PP or PEOP. Different parameters were evaluated, such as lipid accumulation and oxidative stress-related markers.

Results

In hepatic cells, expression of peroxisome proliferator-activated receptors (PPARs) and of stearoyl-CoA desaturase 1 (SCD-1) were assessed as index of lipid metabolism. In endothelial cells, expression of intercellular adhesion molecule-1 (ICAM-1), activation of nuclear factor kappa-B (NF-kB), release of nitric oxide (NO), and wound-healing rate were assessed as index of inflammation.

Conclusion

PEOP extract ameliorated hepatic lipid accumulation and lipid-dependent oxidative unbalance, thus showing potential applications as therapeutic agent tuning down hepatosteatosis and atherosclerosis.

KEYWORDS

Non-alcoholic fatty liver disease; atherosclerosis; olive pomace; polyphenolic compound; oxidative stress.

52 ## 1. INTRODUCTION

53 The critical role of diet on the pathogenesis of obesity and obesity-associated disorders has
54 been well-established. Non-alcoholic fatty liver disease (NAFLD) is the most common
55 disease associated to obesity [1][2], and is correlated with type 2 diabetes and
56 cardiovascular risk [3]. NAFLD defines a wide spectrum of liver diseases which
57 encompasses simple steatosis (NAFL), non-alcoholic steatohepatitis (NASH), cirrhosis,
58 and even hepatocellular carcinoma [4]. Steatosis consists of excess fat accumulation,
59 mainly triglycerides (TGs), within cytosolic lipid droplets (LDs) [5]. Progression of NAFL
60 to NASH is sustained by oxidative stress resulting from reactive oxygen species (ROS)
61 deriving from fat catabolism [6][7]. Oxidative stress, in fact, activates inflammatory
62 signaling pathways such as that sustained by the nuclear factor kappa-B (NF-κB), a
63 transcription factor of the inflammatory response also involved in liver diseases [8].

64 Olive oil, the main fat source of the Mediterranean diet, is an important contributor to
65 human health in the Mediterranean area [9]. The beneficial effects of olive oil have
66 historically been attributed to oleic acid which may decrease LDL-cholesterol and LDL
67 oxidation (a key step in atherosclerosis) [10][11][12][13]. Additional effects of olive oil
68 include protection against heart disease and insulin resistance [14]. Other potentially
69 beneficial components of Mediterranean diet are polyphenols (PP), found primarily in
70 plants and foods including tea, olive oil, red wine. PP appear to prevent several diseases
71 including cardiovascular diseases [15][16]. Mechanisms involve the activation of several
72 pathways governing immunomodulatory and vasodilatory properties [17].

73 The antioxidant activity is the most relevant biological activity of PP which protect cells
74 against many kinds of ROS. However, PP play other biological actions that are as yet
75 poorly understood; in particular the hepatoprotective potential has been reported for some
76 of them [18][19]. A plethora of studies have investigated the possible medical efficacy of
77 plant PP *in vitro*, in cell cultures, in model organisms and, to a lesser extent, in humans.

78 A solid waste from olive oil production is the olive pomace, one of the most widespread
79 agro-industrial by-products in Mediterranean area. Thus, olive oil producers seek
80 alternative uses for olive pomace such as extraction of antioxidant compounds, conversion
81 to a renewable fuel source, and extraction of residual oil.

82 Free fatty acids (FAs) are the major mediators of liver steatosis; in fact, patients with
83 NAFLD have elevated levels of circulating FAs. The stearoyl-CoA desaturase 1 (SCD-1)
84 catalyzes the synthesis of monounsaturated fatty acids (MUFA) from saturated ones (SFA)
85 [20]. FAs are esterified to TGs and stored in cytosolic LDs as protection against their
86 toxicity, or alternatively, FAs are metabolized through β-oxidation in mitochondria and
87 peroxisomes, and through ω–oxidation in the endoplasmic reticulum (ER) with consequent
88 ROS production. Lipid metabolism is regulated by peroxisome proliferator-activated
89 receptors (PPARs) [21]. Uptake of FAs into hepatocytes and their oxidation is regulated
90 mainly by PPARα isoform, while the anabolic esterification and conversion to TGs by
91 PPARγ, whose expression typically increases in NAFLD [22][23].

92 Both hepatocytes and endothelial cells participate in progression of fatty liver disease, in
93 fact, obesity-related metabolic disorders cause endothelial dysfunction. Endothelium is a
94 crucial blood–tissue interface controlling energy supply according to organ needs, as it is
95 the first rate-limiting step in the utilization of long-chain FAs as fuels. Endothelial cells
96 play regulatory functions through releasing various factors including nitric oxide (NO) and
97 ROS [24]. In the liver, the endothelial cells of sinusoids act in fibrosis development by
98 sustaining wound healing response and inflammation [25][26]. Wound healing process
99 depends on endothelial cell migration which is mediated by the intercellular adhesion
100 molecule-1 (ICAM-1) on the plasma membrane. [27].

101 A Mediterranean diet seems to reduce hepatic steatosis in patients with NAFLD, although
102 the mechanisms are still unclear [28][29]. Here we investigated the effects and possible
103 beneficial action of plant PP in terms of lipid-loading and fat-induced oxidative stress in
104 both hepatic and endothelial cells. We used a mixture of polyphenols extracted from olive
105 pomace (PEOP) and, for comparison, the main single phenolic compounds present in the
106 extract (tyrosol, apigenin, oleuropein, coumaric and caffeic acid). The lipid-lowering
107 activity of oleuropein has been widely described*: (i)* oleuropein decreased number and size
108 of LDs and reduced TG accumulation in HepG2 cells [30]; *(ii)* oleuropein both
109 counteracted lipid accumulation in a mouse model of NAFLD [31], and reversed the diet-
110 induced increase in liver weight in mice [32]. Tyrosol seems to protect HepG2 cells against
111 oxidant injury [33]. Apigenin plays general antioxidant and antiproliferative actions [34].

112 Coumaric acid showed anti-adipogenic effect and suppressed dyslipidemia, hepatosteatosis
113 and oxidative stress in obese rats [35]. Caffeic acid inhibited toxin-induced liver injury and
114 plays lipid lowering effects *in vitro* [36].

115 Here, we used rat hepatoma FaO cells exposed to a mixture of oleate/palmitate that
116 represent a reliable *in vitro* model for hepatic steatosis widely employed in previous
117 studies of our group [37][38]. As FAs seem to play also direct effects on oxidative stress of
118 vascular endothelium [39], we used also human endothelial HECV cells exposed to FAs
119 that could be compared to *in vivo* atherosclerosis, as endothelial damage is typically
120 observed in metabolic syndrome [40].

121 The results showed that PEOP ameliorated lipid accumulation in hepatic cells and reduced
122 lipid-dependent oxidative unbalance in both hepatic and endothelial cells thus showing
123 potential applications as therapeutic agents.

124

125 **2. MATERIALS AND METHODS**

126 ***2.1 Chemicals***

127 All chemicals, unless otherwise indicated, were supplied by Sigma-Aldrich Corp. (Milan,
128 Italy).

129 ***2.2 Polyphenol extraction and quantification***

130 For the extraction of polyphenols from olive pomace of Taggiasca cultivar we used an
131 innovative and green extraction technique using a high pressure and temperature (HPTE)
132 reactor (model 4560, PARR Instrument Company, Moline, USA). Extraction temperature
133 (180°C) and time (90 min) were selected based on previous work [41]. According to latest
134 studies [42][43] 5gr of dried olive pomace was mixed in 50mL of ethanol:water solution
135 with different ethanol percentage. The extraction vessel was free of oxygen by purging
136 nitrogen inside the chamber and closed hermetically. The pressure inside the reactor
137 reached 25 bar. After extraction, the liquid phase was separated from the mixture by
138 centrifugation (6000×g for 10 min) and then stored at -20°C. Before cellular treatments,

the polyphenolic extract of olive pomace (PEOP) was concentrated ten times using a rotary evaporator (Laborota, Heidolph, Germany).

Total polyphenolic content (TPC) was measured using Folin-Ciocalteu assay [44] and was expressed as milligrams of caffeic acid equivalents (CAE) per milliliter of extractive solvent (mg_{CAE}/mL). Single phenolic compounds were detected following the methodology described by Paini et al. [42] using a HPLC (Hewlett Packard, 1100 Series, Palo Alto, CA, USA) coupled with a DAD detector.

2.3 Cell culture and treatments

FaO cells (European Collection of Authenticated Cell Cultures, Sigma-Aldrich) are a rat hepatoma cell line maintaining hepatocyte-specific markers [45]. Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C in Coon's modified Ham's F12 medium supplemented with L-Glutamine and 10% foetal calf serum (FCS). HECV cells (Cell Bank and Culture-GMP-IST-Genoa, Italy) are a human endothelial cell line isolated from umbilical vein; they were grown at 37°C in Dulbecco's modified Eagle's medium High Glucose (D-MEM) supplemented with L-Glutamine and 10% FCS. For treatments, cells were grown until 80% confluence, then incubated overnight in serum-free medium with 0.25% bovine serum albumin (BSA). To mimic *in vitro* the effect of a high fat diet, cells were treated for 3 h with a mixture of oleate/palmitate at a final concentration of 0.75 mM (2:1 molar ratio). Thereafter, 'steatotic' cells (OP) were incubated for 24h in the absence or in the presence of single PP (10 µg/mL tyrosol, 13 µg/mL apigenin, 50 µg/mL oleuropein, 25µM coumaric acid, 25µM caffeic acid) or, alternatively, in the presence of PEOP at two different concentrations (0.05 and 0.1 mg_{CAE}/mL). Stock solution of PEOP 10mM in ethanol/water was diluted with the culture medium to the working concentration.

2.4 Protein quantification

The protein content was determined by the bicinchoninic acid (BCA) method using BSA as a standard [46]. All measurements were performed using a Varian Cary50 spectrophotometer.

166 ***2.5 Quantification of triglycerides***

167 At the end of the treatments, FaO and HECV cells were scraped and centrifuged at
168 14,000xg for 3 min. After cell lysis, lipids were extracted in chloroform/methanol (2:1)
169 then chloroform was evaporated [37]. In each extract, TG content was determined by
170 spectrophotometric analysis using the 'Triglycerides liquid' kit (Sentinel, Milan, Italy).
171 Values were normalized for the protein content determined by the bicinchoninic acid
172 (BCA). Data are expressed as percent TG content relative to controls.

173 ***2.6 ROS production and lipid peroxidation measurement***

174 The oxidation of the cell-permeant 2'-7' dichlorofluorescin diacetate (DCF-DA, Fluka,
175 Germany) to 2'-7'dichlorofluorescein (DCF) is used for quantifying *in situ* the production
176 of H_2O_2 and other ROS [47]. Stock solution of DCF-DA (10mM in DMSO) was prepared
177 and stored at -20°C in the dark. At the end of treatment, cells were scraped and gently spun
178 down (600xg for 10 min at 4°C). After washing, cells were loaded with 10μM DCF-DA in
179 PBS for 30 min at 37°C in the dark. Then, cells were centrifuged, suspended in PBS and
180 the fluorescence was measured fluorometrically (λex=495 nm; λem=525 nm). All
181 measurements were performed in a LS50B fluorimeter (Perkin Elmer, USA) at 25°C using
182 a water-thermostated cuvette holder.

183 Lipid peroxidation was determined spectrophotometrically through the thiobarbituric acid
184 reactive substances (TBARS) assay which is based on the reaction of malondialdehyde
185 (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) [48]. Briefly, 1 vol.
186 of cell suspension was incubated for 45 min at 95°C with 2 vol. of TBA solution (0.375%
187 TBA, 15% trichloroacetic acid, 0.25 N HCl). Then, 1 vol. of N-butanol was added and the
188 organic phase was read at 532 nm in a Varian Cary 50 Bio UV-VIS spectrophotometer
189 (Agilent, Milan, Italy) at 25°C using Peltier-thermostated cuvette holder. The MDA level
190 was expressed as pmol MDA/ml/mg protein.

191 ***2.7 Oil-Red O staining***

192 Neutral lipids were visualized using the selective Oil-RedO (ORO) dye [49]. Briefly, after
193 fixing in 4% paraformaldehyde, cells were washed with PBS, stained for 20 min with 0.3%

194 ORO solution prepared from a stock 0.5% in isopropanol and diluted in water. After
195 washing with distilled water, slides were examined by Leica DMRB light microscope
196 equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany).

197 ***2.8 Measurement of Nitrite/Nitrate (NOx) Levels***

198 NO production was measured by spectrophotometric measurement of the end products,
199 nitrites and nitrates, using the Griess reaction [50]. After treatments, nitrite accumulation
200 (µmol $NaNO_2$/mg sample protein) was calculated against a standard curve of sodium nitrite
201 ($NaNO_2$). All spectrophotometric analyses were carried out at 25°C recording absorbance
202 at 540nm with a Varian Cary 50 spectrophotometer. Data are means ± S.D. of at least four
203 independent experiments.

204 ***2.9 RNA extraction and real-time qPCR***

205 RNA was isolated using Trizol reagent, cDNA was synthesized and quantitative real-time
206 PCR (qPCR) was performed in quadruplicate using 1x IQTMSybrGreen SuperMix and
207 Chromo4TM System apparatus (Biorad, Milan, Italy) [37]. The relative quantity of target
208 mRNA was calculated by the comparative Cq method using glyceraldehyde 3-phosphate
209 dehydrogenase (GAPDH) as housekeeping gene, and expressed as fold induction with
210 respect to controls [51]. Primer pair sequence have been previously reported
211 [37][38][52][53].

212 ***2.10 Western blotting***

213 Protein levels of NF-kB p65 was assessed by western blot analysis. Briefly, the cellular
214 pellet was suspended in 400µl ice-cold Buffer A (20mM Tris HCl pH 7.8, 50mM KCl,
215 10µg/ml Leupeptin, 0.1mM Dithiothreitol-DTT, 1mM phenylmethanesulfonyl fluoride-
216 PMSF); then 400 µl Buffer B (Buffer A plus 1.2% Nonindet P40) was added. The
217 suspension was vortex-mixed for 10 sec; after centrifugation (14000xg for 30 sec, 4°C) the
218 supernatant was discarded and the nuclear pellet was washed with 400 µl Buffer A and
219 centrifuged. The nuclear pellet was suspended in 100 µl Buffer B, mixed thoroughly in ice
220 for 15 min and finally centrifuged (14000xg for 20 min, 4°C). The supernatant containing
221 the nuclear extracts was collected and the protein content was measured by BCA method.
222 About 40µg proteins were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide

223 gel electrophoresis (SDS-PAGE) [54]. Membrane was blocked for 1h in 5% fat-free
224 milk/PBS (pH 7.4) and probed using rabbit anti-human NF-kB p65 (SC-109) antibody
225 supplied by Santa Cruz Biotechnology (DBA, Milan, Italy). Membranes were incubated
226 overnight at 4°C with primary antibody in PBST buffer (PBS with 0.1% Tween 20) [55]
227 washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse
228 IgG (Sigma-Aldrich) in PBST for 1h at room temperature. Immune complexes were
229 visualized using an enhanced chemiluminescence western blotting analysis system (Bio-
230 Rad ChemiDoc XRS System). Films were digitized and band optical densities were
231 quantified against the actin band using a computerized imaging system and expressed as
232 Relative Optical Density (ROD, arbitrary units). ROD of each band was expressed as
233 percentage respect to control.

234 ***2.11 Wound Healing assay***

235 The migration of HECV was examined using the wound healing assay. The cells were
236 seeded on 35 × 10 mm tissue culture dishes and incubated for 24 h or until confluence was
237 reached. After 3h of incubation with oleate/palmitate mixture, the cells were scrapped
238 horizontally and vertically with a P100 pipette tip (Eppendorf AG, Hamburg, Germany)
239 and two views on the cross were photographed on each well attached to the microscope at
240 4x magnification. The medium was replaced with fresh medium in the absence or presence
241 of PEOP at two different concentrations (0.05 and 0.1 mg_{CAE}/mL). Set of images were
242 acquired at 3, 6 and 24 h. To determine the migration of HECV, the images were analysed
243 using ImageJ free software (http://imagej.nih.gov/ij/). Percentage of the closed area was
244 measured and compared with the value obtained before treatment. An increase of the
245 percentage of closed area indicated the migration of cells. Data are means ± S.D. of at least
246 three independent experiments.

247 ***2.12 Statistical analysis***

248 RNA and protein data are expressed as means ± S.D. of at least four independent
249 experiments in triplicate. Statistical analysis was performed using ANOVA with Tukey's
250 post-test (GraphPad Software, Inc., San Diego, CA, USA).

251 **3. RESULTS**

252 ***3.1 Total polyphenol content of the extracts***

253 Polyphenol extraction was performed using HPTE. Increasing the ethanol percentage in the
254 extraction solvent from 0 to 75%, (v/v) resulted in an increase in TPC from 2.1±0.1 to
255 6.0±0.1 mg_{CAE}/mL, respectively, thus confirming the synergistic effect of water and
256 ethanol as extraction solvent [42][43]. The TPC reached with 75% ethanol is close to the
257 TPC (5.8±0.1 mg_{CAE}/mL) obtained using ethanol:water of 50:50 (v/v). Taking into account
258 both TPC value and the necessity of reducing the amount of organic solvent in the medium
259 we selected the extract composed of 50% (v/v) ethanol as solvent. HPLC-DAD analysis
260 allowed to quantify the main phenolic compounds in the extract (Table 1). Oleuropein
261 presented the highest concentration (0.49 mg/L) in the extract, followed by tyrosol (0.10
262 mg/mL).

263

264 ***3.2 Effects of polyphenols on lipid accumulation in hepatic and endothelial cells***

265 Exposure of cells to FAs was used to model *in vitro* what is occurring *in vivo* in different
266 tissues during high fat feeding and/or obesity. Both FaO and HECV cells were overloaded
267 of lipids by exposure to oleate/palmitate mixture (0.75 mM) for 3h. Then, cells were
268 treated for 24h with PEOP extract (0.05 or 0.1 mg_{CAE}/mL) or with the single polyphenols
269 Apigenin (Api, 13 µg/mL), Caffeic acid (Caf, 25µM), Coumaric acid (Cou, 25µM),
270 Oleuropein (Ole, 50 µg/mL), Tyrosol (Tyr, 10 µg/mL) as comparison. MTT assay was
271 performed to exclude toxic effects of the treatments (data not shown).

272 The intracellular TG content was quantified in control (C) and steatotic cells incubated in
273 the absence (OP) or in the presence of the different polyphenols. In both FaO and HECV
274 cells (Fig. 1A-B), we observed a significant increase in TG content with respect to control
275 (+136% for FaO and +177% for HECV; $p \leq 0.001$ for both). In steatotic FaO cells, Tyrosol
276 and Oleuropein significantly reduced the TG content (-31% and -28%, respectively, with
277 respect to steatotic cells; $p \leq 0.001$), while no significant effects were observed with
278 Apigenin, Caffeic and Coumaric acids (Fig. 1A). A significant lipid-lowering action was
279 observed also with PEOP extract (-32% for 0.05 mg_{CAE}/mL and -21% for 0.01 mg_{CAE}/mL
280 doses with respect to steatotic cells; $p \leq 0.01$ and $p \leq 0.05$, respectively). Conversely, no
281 lipid-lowering action could be appreciated in steatotic HECV cells as a response to both
282 single PP or PEOP extract (Fig. 1B). Surprisingly, the higher PEOP concentration (0.1

283 mg_{CAE}/mL) led to a further increase in TG content in HECV cells (+76% with respect to
284 steatotic cells; p≤0.001). PEOP did not modify the TG content in both control FaO and
285 HECV cells (data not shown).
286 Cytosolic LDs were visualized by ORO staining (Fig.1C-D). In FaO cells, the number and
287 size of LDs increased markedly in steatotic cells (OP) compared to control and decreased
288 upon incubation with PEOP (Fig.1C). Also steatotic HECV cells (OP) showed an evident
289 increase in size and number of LDs, that was not modified by PEOP (Fig.1D).

290 ***3.3 Effects of polyphenols on oxidative stress in hepatic and endothelial cells***

291 As an indicator of oxidative stress lipid peroxidation was assessed by TBARS assay. The
292 MDA level (Fig. 2A) increased in steatotic FaO cells (+122% compared to control;
293 p≤0.001), and significantly decreased upon exposure to Apigenin (-33%, p≤0.05), Caffeic
294 acid (-59%, p≤0.001), Coumaric acid (-43%, p≤0.001) or Oleuropein (-30%, p≤0.05),
295 whereas no changes were observed with Tyrosol. The MDA level decreased also when
296 steatotic FaO cells were exposed to both 0.05 and 0.1 mg_{CAE}/mL doses of PEOP (-35%
297 and-45% with respect to steatotic cells; p≤0.01 and p≤0.001, respectively) (Fig. 2A). Also
298 in HECV cells, the MDA level (Fig. 2B) was increased upon lipid-loading (+131% with
299 respect to control; *p*≤0.001) and the single PP counteracted this effect leading to a decrease
300 in MDA level of -43% (*p*≤0.001) for Apigenin, -39% (*p*≤0.01) for Caffeic acid, -35%
301 (*p*≤0.01) for Coumaric acid, -39% (*p*≤0.001) for Oleuropein, and -54% (*p*≤0.001) for
302 Tyrosol compared to steatotic cells. The MDA level decreased also with both 0.05 and 0.1
303 mg_{CAE}/mL doses of PEOP (-39% and -42% with respect to steatotic cells, respectively;
304 p≤0.001) (Fig. 2B).
305 Fluorimetric analysis allowed to assess *in situ* the ROS production, mainly hydrogen
306 peroxide, as a response to PEOP (Fig. 2C). Steatotic FaO cells treated with PEOP at both
307 doses showed a significant decrease in DCF florescence with respect to steatotic cells used
308 as control (-28% and -38%; p≤0.05 and p≤0.01, respectively). Also steatotic HECV cells
309 treated with PEOP showed a significant DCF decrease with respect to steatotic cells (-27%
310 and -33%; p≤0.01 p≤0.001, respectively) (Fig. 2D).
311 In control FaO and HECV cells, neither the MDA level or DCF signal were affected by PP
312 and PEOP (data not shown).
313

3.4 Effects of polyphenols on hepatic lipid metabolism

Hepatic lipid metabolism is under the control of PPARs; PPARα and PPARγ are the most abundant isoforms in FaO cells (PPARα>PPARγ) [37]. Expression of PPARα mRNA did not significantly change in steatotic cells compared to control (Fig. 3A); incubation with the highest dose of PEOP (0.1 mg_{CAE}/mL) resulted in a significant decrease in PPARα expression (-42% with respect to steatotic cells; $p \leq 0.05$). On the contrary, a significant up-regulation of PPARγ expression was observed upon lipid-loading (1.88 fold induction with respect to control; $p \leq 0.05$), and PEOP at the highest concentration led to a further increase in mRNA transcripts (+38% with respect to steatotic cells, $p \leq 0.05$) (Fig 3A).

SCD-1 catalyses synthesis of unsaturated FAs. In steatotic FaO cells, SCD-1 mRNA expression decreased with respect to control (0.54 fold induction; $p \leq 0.01$) and PEOP at both concentrations did no change significantly SCD-1 expression (Fig.3B).

The lipid-lowering action of PEOP might be sustained by stimulation of oxidative and/or secretory pathways. With regard to mitochondrial β-oxidation, CPT1 expression was significantly up-regulated upon lipid-loading (2.17 fold induction with respect to control; $p \leq 0.05$) (Fig.3C). PEOP at both concentrations led to a further up-regulation of CPT1 expression (+86% and +156% with respect to steatotic cells; $p \leq 0.01$ and $p \leq 0.001$, respectively). Moreover, in steatotic FaO cells we observed a significant increase in extracellular TG content with respect to control (+21%; $p \leq 0.05$). Exposure to the highest PEOP concentration (0.1 mg_{CAE}/mL) resulted in a further increase in TG secretion (+20%; $p \leq 0.01$) with respect to steatotic cells (Fig.3D).

3.5 Effects of polyphenols on endothelial function

NO is a major modulator of endothelial cell activity. In steatotic HECV cells, we observed a significant increase in NO release with respect to control (+88%; $p \leq 0.001$), that was counteracted by both concentrations of PEOP (-61% for 0.05 mg_{CAE}/mL, and -66% for 0.1 mg_{CAE}/mL, with respect to steatotic cells; $p \leq 0.001$) (Fig. 4A).

Similar results were observed for NF-κB activation that mediates inflammatory response. Steatotic HECV cells showed increased NF-κB p65 level of with respect to control (+28%; $p \leq 0.01$); this increase was completely reverted by both concentrations of PEOP (-21% for

344 0.05 mg_{CAE}/mL, and -34% for 0.1 mg_{CAE}/mL, with respect to steatotic cells; $p \leq 0.01$ and
345 $p \leq 0.001$, respectively) (Fig. 4B).
346 Expression of ICAM-1 mRNA did not change significantly in steatotic HECV cells
347 compared to control (Fig. 4C), but incubation with the highest PEOP concentration (0.1
348 mgCAE/mL) resulted in a significant increase (+164% with respect to steatotic cells;
349 $p \leq 0.001$). On the other hand, expression of MT-2A did not change significantly for all
350 treatments tested here (Fig. 4C).
351 The PEOP effects on migrating ability of HECV cells was evaluated using the Wound
352 Healing assay (Fig.4D-E). No significant differences in cell migration rate were observed
353 at short times after the scratch (3 and 6 h), whereas at a longer time (24 h) the steatotic
354 cells showed a wound width (54%) similar to that of controls (57%). Both doses of PEOP
355 induced a significant slowdown of cell migration with a wound width larger to that of
356 controls (71% for 0.05 mg_{CAE}/mL, and 74% for 0.1 mg_{CAE}/mL).
357

358 **4. DISCUSSION**

359 This study shows that a mixture of polyphenols extracted from olive pomace (PEOP) may
360 counteract steatosis in a cellular model of NAFLD. At least in this model, the lipid-
361 lowering effect is sustained by modulation of lipid metabolism and packaging. Notably,
362 PEOP extract also ameliorated lipid-dependent oxidative unbalance in endothelial cells,
363 All together, our data suggest that PEOP might play a protective role as possible
364 therapeutic agents acting on hepatosteatosis and atherosclerosis.
365 In the last decades, many beneficial properties of plant polyphenols have been identified by
366 an increasing body of studies performed on cultured cells, model animals and humans.
367 [55]. For example, a randomized crossover trial conducted in 200 men showed a dose-
368 response effect in raising HDL-cholesterol and lowering oxidized LDL which was more
369 evident for virgin olive oil (high in polyphenols) than for olive oil (low in polyphenols)
370 [57] [58].

371 With regard to hepatocytes, the present results show that PEOP extract ameliorated lipid
372 accumulation and lipid-dependent oxidative unbalance in steatotic cells. In fact, *in situ* and
373 *in vitro* analyses of LD staining and TG quantification, respectively, showed a significant

374 reduction in fat accumulation in cells treated with PEOP without effects on cell viability.
375 As comparison, the effects of the single PP composing the PEOP extract have been
376 assessed showing different effect depending on the polyphenol molecule. Anti-obesity
377 effects of diets rich in PP have been previously described in both patients and animal
378 models [32][59], but they were mainly attributed to the ability of polyphenols to interact at
379 first with adipose tissue which then releases molecules acting as a second step on liver.
380 Therefore, a first result of the present study is the demonstration that the lipid-lowering
381 action of PP depend, at least in part, on a direct action on hepatic cells rather than on
382 adipose tissues.

383 Dietary polyphenols may prevent steatosis development through the following
384 mechanisms: (*i*) decreased lipogenesis; (*ii*) increased lipolysis; (*iii*) attenuation of
385 inflammatory responses and oxidative stress. Our results clearly indicate that the lipid-
386 lowering action of PEOP extract on steatotic hepatocytes is, at least in part, sustained by
387 stimulation of TG secretion. This is confirmed by the modulation of PPARγ expression
388 which increased upon exposure to the highest PEOP concentration; in fact PPARγ
389 promotes esterification and conversion of FAs to TGs and their packaging as VLDL.
390 Moreover, PPARγ has anti-inflammatory effects by attenuating secretion of
391 proinflammatory cytokines (including IL-1β and TNF-α) [60]. On the other hand, the
392 reduced expression of PPARα upon exposure to PEOP suggests that the extract might
393 reduce the FA uptake from the external medium. At the same time, PEOP extract seems to
394 reduce lipid accumulation by stimulating mitochondrial β-oxidation, as indirectly
395 suggested by the up-regulation of CPT-1 expression. On the other hand, PEOP did not
396 influence expression of SCD-1 that converts SFA to MUFA which are less toxic for the
397 cell. It has to be noted that the principal product of SCD-1 activity is oleic acid, but in our
398 *in vitro* model of NAFLD we supplied large amount of exogenous oleate and this fact can
399 explain the reduced expression of SCD-1 in steatotic cells.

400 Exposure of FaO cells to PEOP significantly reduced the ROS level and the lipid
401 peroxidation that are typically increased in steatotic hepatocytes, and that could lead to
402 immunological dysfunction triggering hepatic fibrosis [61]. It has to be noted that, whereas
403 the antioxidant effect was more marked for the highest PEOP concentration, the anti-

404 steatotic effect was more evident for the lowest dose. However, a non-monotonic response
405 to increasing concentrations of a substance has been widely described in endocrinology
406 and pharmacology.

407 Endothelium damage is one of the main metabolic abnormalities observed in NAFLD [40].
408 Endothelial cells form the sinusoid wall separating hepatocytes from blood, and act in
409 hepatic inflammation through their involvement in adhesion molecule-mediated
410 recruitment of leukocytes [62]. Two major effector systems implicated in the vascular
411 alterations associated with inflammation and atherosclerosis involve the generation of ROS
412 and NO. Here, we loaded HECV cells of FAs to mimic what is occurring *in vivo* during
413 high fat feeding. Interestingly, exposure of lipid-loaded HECV cells to PEOP did not
414 reduce lipid accumulation, rather increased it at the highest dose. Consistent with these
415 effect on fat accumulation, the highest dose of PEOP led to increased expression of the
416 adhesion molecule ICAM-1 on the cell membrane. ICAM-1 expression is typically
417 increased by inflammatory mediators [63] such as those produced by excess fat
418 accumulation.

419 As a response to lipid-loading, endothelial cells released NO and produced ROS thus
420 triggering pro-inflammatory events. NO modulates leukocyte-endothelial cell interactions
421 by: *(i)* affecting leukocyte adhesion in the microcirculation through activation of NF-κB;
422 *(ii)* inhibiting platelet aggregation; *(iii)* protecting cells from oxidative stress through its
423 interaction with superoxide which acts as a proadhesive molecule [64]. PEOP extract
424 significantly reduced the ROS level and the ROS-dependent lipid peroxidation, as well as
425 the NO release. NO is a major vasodilative substance produced by the endothelium; at low
426 levels NO is a second messenger playing beneficial effects, while high levels of NO may
427 cause detrimental effects through its reaction with superoxide anion for example [65]. NO
428 also influences endothelial cell migration which is responsible for the wound healing
429 process that, together with inflammation, sustain the progression of fatty liver towards
430 fibrosis [26]. Accordingly with their effects on NO release, we observed that PEOP
431 inhibited the rate of endothelial cell migration. The rate of cell migration was not modified
432 upon fat-loading in HECV cells, whereas it was significantly slowed by exposure to PEOP.

433 The translational value of this study should not be overlooked since NAFLD and
434 atherosclerosis share common metabolic abnormalities and NAFLD appears to be
435 independently associated with cardiovascular disease.

436 In conclusion, we show here that PEOP have potential beneficial on two key metabolic
437 disorders of lipid metabolism, i.e. ameliorated hepatic lipid accumulation and endothelial
438 and hepatic lipid-dependent oxidative unbalance. Notably, PEOP may lead to a novel
439 nutraceutical formulation, in which the nutritive characteristics of food can be enriched
440 with the health benefits related to polyphenols consumption. Further studies should extend
441 the seminal observations in the present study and test the beneficial effects of certain
442 concentration of polyphenols on both liver and vascular morphology and function.

443

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449 2013-16 (PP).

450 **CONFLICT OF INTEREST STATEMENT**

451 The authors declare that they have no conflict of interest.

452

453 ***Table 1*: ** Concentration (mg/mL) of single phenolic compounds in olive pomace extract,
454 analysed by high-performance liquid chromatography.
455

Phenolic compound	Concentration (mg/mL)
Tyrosol	0.10
Caffeic acid	0.03
Coumaric acid	0.03
Oleuropein	0.49
Apigenin	0.01

456

457 **FIGURE LEGENDS**

458 ***FIGURE 1: Effects of polyphenols on lipid accumulation in hepatic and endothelial***
459 ***cells***

460 In FaO **(A)** and in HECV **(B)** cells, TG content was quantified by spectrophotometric assay
461 in control and steatotic cells incubated in the absence (OP) or in the presence of the single
462 polyphenols Apigenin (Api, 13 µg/mL), Tyrosol (Tyr, 10 µg/mL), Oleuropein (Ole, 50
463 µg/mL), Coumaric (Cou , 25µM), Caffeic (Caf, 25µM) acid and of the PEOP extract at two
464 concentrations (0.05 and 0.1 mg_{CAE}/mL) for 24h. Data are expressed as percent TG content
465 relative to control and normalized for total proteins. Neutral lipid accumulation was
466 assessed i*n situ* in ORO-stained FaO **(C)** and HECV **(D)** cells incubated in the absence
467 (OP) or in the presence of PEOP extract (0.05 and 0.1 mg_{CAE}/mL). (magnification 10x;
468 Bar: 50 µm; magnification 20x and 40x; Bar: 20 µm). Values are mean ± S.D from a least
469 three independent experiments. ANOVA followed by Tukey's test was used to assess the
470 statistical significance between groups Significant differences are denoted by symbols: C
471 *vs* OP ***p≤0.001 and OP *vs* single polyphenols or PEOP 0.05 and 0.1 mg_{CAE}/mL ###
472 p≤0.001, ##p≤0.01, #p≤0.05)**.**

473

474 ***FIGURE 2: Effects of polyphenols on lipid peroxidation and intracellular ROS***
475 ***production in hepatic and endothelial cells***

476 In both FaO **(A)** and in HECV **(B)** cells, the intracellular level of MDA (pmol MDA/ml x
477 mg of sample protein) was quantified by TBARS assay in control and steatotic cells
478 incubated in the absence (OP) or in the presence of the single polyphenols Apigenin (Api),
479 Tyrosol (Tyr), Oleuropein (Ole), Coumaric (Cou), Caffeic (Caf) acid and of the PEOP
480 extract at two concentrations (0.05 and 0.1 mg_{CAE}/mL). Data are expressed as percentage
481 values with respect to controls and normalized for total protein. The intracellular level of
482 ROS was quantified by spectrofluorimeter assay of DCF-stained FaO **(C)** and HECV cells
483 **(D)** in the absence (OP) or the presence of PEOP extract (0.05 and 0.1 mg_{CAE}/mL). Data
484 are expressed as percent Mean Fluorescence Intensity (MFI) relative to steatotic cells and
485 normalized for total proteins. Values are mean ± S.D from at least three independent
486 experiments. ANOVA followed by Tukey's test was used to assess the statistical
487 significance between groups. Significant differences are denoted by symbols: C *vs* OP
488 ***p≤0.001 and OP *vs* single polyphenols or PEOP 0.05 and 0.1 mg_{CAE}/mL ### p≤0.001,
489 ##p≤0.01, #p≤0.05)**.**

490

491 ***FIGURE 3: Effects of polyphenols on hepatocyte function***

492 The mRNA expression of PPARα and PPARγ **(A)**, of SCD1 **(B)** and of CPT1 **(C)** was
493 quantified in control (C) and steatotic FaO cells incubated in the absence (OP) or in the
494 presence of PEOP extract (0.05 and 0.1 mg_{CAE}/mL) by qPCR. GAPDH was used as the
495 internal control for quantifying gene expression; data expressed as fold induction with
496 respect to controls. (**D)** Extracellular TG content was quantified in the medium by
497 spectrophotometric assay. Data are expressed as percent TG content relative to control and
498 normalized for total proteins. Values are mean ± S.D from at least three independent
499 experiments. ANOVA followed by Tukey's test was used to assess the statistical
500 significance between groups. Significant differences are denoted by symbols: C vs OP
501 ***p≤0.001, **p≤0.01, *p≤0.05 OP *vs* PEOP 0.05 and 0.1 mg_{CAE}/mL mL ### p≤0.001,
502 ##p≤0.01, #p≤0.05).

503 ***FIGURE 4: Effects of polyphenols on endothelial cell function***

504 **(A)** NO production was quantified in the medium of control (C) and steatotic HECV cells
505 incubated in the absence (OP) or in the presence of PEOP extract (0.05 and 0.1 mg_{CAE}/mL)
506 by Griess reaction and normalized for protein content **(B)** Densitometric analysis of
507 nuclear NF-kB/p65 was evaluated by Western blotting; β-actin was the protein loading
508 control used as housekeeping gene for normalization and data are expressed as percentage
509 values with respect to controls. **(C)** The mRNA expression of ICAM-1 and MT2A was
510 quantified by qPCR; GAPDH was used as the internal control for quantifying gene
511 expression and data expressed as fold induction with respect to controls. **(D)** Cell migration
512 measured by the T scratch assay photographed at 6 and 24 h incubation with PEOP extract
513 (0.05 and 0.1 mgCAE/mL). **(E)**.Graphs representing the percentage of the closed area as
514 compared to time=0. T scratch assay representative images are also shown Values are
515 mean ± S.D from at least three independent experiments. ANOVA followed by Tukey's
516 test was used to assess the statistical significance between groups. Significant differences
517 are denoted by symbols: C vs OP ***$p \leq 0.001$, **$p \leq 0.01$, OP *vs* PEOP 0.05 and 0.1
518 mg_{CAE}/mL mL ### $p \leq 0.001$, ##$p \leq 0.01$)**.**
519

520 **References**

521 [1] Loomba R, Sanyal AJ. The global NAFLD epidemic. Nat Rev Gastroenterol
522 Hepatol 2013;10:686–90. doi:10.1038/nrgastro.2013.171.

523 [2] Huang YY, Gusdon AM, Qu S. Nonalcoholic fatty liver disease: molecular
524 pathways and therapeutic strategies. Lipids Health Dis 2013;12:171.
525 doi:10.1186/1476-511X-12-171.

526 [3] Anstee QM, Targher G, Day CP. Progression of NAFLD to diabetes mellitus,
527 cardiovascular disease or cirrhosis. Nat Rev Gastroenterol Hepatol 2013;10:330–44.
528 doi:10.1038/nrgastro.2013.41.

529 [4] Cohen JC, Horton JD, Hobbs HH. Human Fatty Liver Disease: Old Questions and
530 New Insights. Science (80-) 2011;332:1519–23. doi:10.1126/science.1204265.

531 [5] Sahini N BJ. Recent insights into the molecular pathophysiology of lipid droplet
532 formation in hepatocytes. Progr Lipid Res 2014:86–112.
533 doi:10.1016/j.plipres.2014.02.002.

534 [6] Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology
535 1998;114:842–5.

536 [7] Rolo AP, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of
537 nonalcoholic steatohepatitis. Free Radic Biol Med 2012;52:59–69.
538 doi:10.1016/j.freeradbiomed.2011.10.003.

539 [8] Hoesel B, Schmid JA. The complexity of NF-κB signaling in inflammation and
540 cancer. Mol Cancer 2013;12:86. doi:10.1186/1476-4598-12-86.

541 [9] Rigacci S, Stefani M. Nutraceutical Properties of Olive Oil Polyphenols. An
542 Itinerary from Cultured Cells through Animal Models to Humans. Int J Mol Sci
543 2016;17. doi:10.3390/ijms17060843.

544 [10] Mata P, Alvarez-Sala LA, Rubio MJ, Nu??o J, De Oya M. Effects of long-term
545 monounsaturated- vs polyunsaturated-enriched diets on lipoproteins in healthy men
546 and women. Am J Clin Nutr 1992;55:846–50.

547 [11] Mensink RP, Katan MB. Effect of a Diet Enriched with Monounsaturated or
548 Polyunsaturated Fatty Acids on Levels of Low-Density and High-Density
549 Lipoprotein Cholesterol in Healthy Women and Men. N Engl J Med 1989;321:436–
550 41. doi:10.1056/NEJM198908173210705.

551 [12] Mensink RP, Katan MB. Effect of dietary fatty acids on serum lipids and
552 lipoproteins. A meta-analysis of 27 trials. Arterioscler Thromb 1992;12:911–9.
553 doi:10.1161/01.ATV.12.8.911.

554 [13] Reaven P, Parthasarathy S, Grasse BJ, Miller E, Steinberg D, Witztum JL. Effects of
555 oleate-rich and linoleate-rich diets on the susceptibility of low density lipoprotein to
556 oxidative modification in mildly hypercholesterolemic subjects. J Clin Invest
557 1993;91:668–76. doi:10.1172/JCI116247.

558 [14] Ryan M, McInerney D, Owens D, Collins P, Johnson a, Tomkin GH. Diabetes and
559 the Mediterranean diet: a beneficial effect of oleic acid on insulin sensitivity,
560 adipocyte glucose transport and endothelium-dependent vasoreactivity. QJM
561 2000;93:85–91. doi:10.1093/qjmed/93.2.85.

562 [15] Owen RW, Giacosa A, Hull WE, Haubner R, Würtele G, Spiegelhalder B, et al.
563 Olive-oil consumption and health: the possible role of antioxidants. Lancet Oncol
564 2000;1:107–12.

565 [16] Visioli F, Poli A, Gall C. Antioxidant and other biological activities of phenols from
566 olives and olive oil. Med Res Rev 2002;22:65–75.

567 [17] Zern TL, Fernandez ML. Cardioprotective Effects of Dietary Polyphenols. J Nutr
568 2005:2291–4.

569 [18] Itoh A, Isoda K, Kondoh M, Kawase M, Watari A, Kobayashi M, et al.
570 Hepatoprotective effect of syringic acid and vanillic acid on CCl4-induced liver
571 injury. Biol Pharm Bull 2010;33:983–7.

572 [19] Poudyal H, Campbell F, Brown L. Olive leaf extract attenuates cardiac, hepatic, and
573 metabolic changes in high carbohydrate-, high fat-fed rats. J Nutr 2010;140:946–53.
574 doi:10.3945/jn.109.117812.

575 [20] Gutiérrez-Juárez R, Pocai A, Mulas C, Ono H, Bhanot S, Monia BP, et al. Critical
576 role of stearoyl-CoA desaturase-1 (SCD1) in the onset of diet-induced hepatic
577 insulin resistance. J Clin Invest 2006;116:1686–95. doi:10.1172/JCI26991.

578 [21] Viswakarma N, Jia Y, Bai L, Vluggens A, Borensztajn J, Xu J, et al. Coactivators in
579 PPAR-Regulated Gene Expression. PPAR Res 2010;2010.
580 doi:10.1155/2010/250126.

581 [22] Martin G, Schoonjans K, Lefebvre AM, Staels B, Auwerx J. Coordinate regulation
582 of the expression of the fatty acid transport protein and acyl-CoA synthetase genes
583 by PPARalpha and PPARgamma activators. J Biol Chem 1997;272:28210–7.

584 [23] Yu S, Matsusue K, Kashireddy P, Cao W-Q, Yeldandi V, Yeldandi A V, et al.
585 Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due
586 to peroxisome proliferator-activated receptor gamma1 (PPARgamma1)
587 overexpression. J Biol Chem 2003;278:498–505. doi:10.1074/jbc.M210062200.

588 [24] Shah V, Haddad FG, Garcia-Cardena G, Frangos JA, Mennone A, Groszmann RJ, et
589 al. Liver sinusoidal endothelial cells are responsible for nitric oxide modulation of
590 resistance in the hepatic sinusoids. J Clin Invest 1997;100:2923–30.
591 doi:10.1172/JCI119842.

592 [25] Braet F, Wisse E. Structural and functional aspects of liver sinusoidal endothelial
593 cell fenestrae: a review. Comp Hepatol 2002;1:1.

594 [26] Connolly MK, Bedrosian AS, Malhotra A, Henning JR, Ibrahim J, Vera V, et al. In
595 hepatic fibrosis, liver sinusoidal endothelial cells acquire enhanced immunogenicity.
596 J Immunol 2010;185:2200–8. doi:10.4049/jimmunol.1000332.

597 [27] Gay AN, Mushin OP, Lazar DA, Naik-Mathuria BJ, Yu L, Gobin A, Smith CW OO. Wound healing characteristics of ICAM-1 null mice devoid of all isoforms of ICAM-1. J Surg Res 2011:1–7. doi:10.1016/j.jss.2011.06.053.

600 [28] Kontogianni MD, Tileli N, Margariti A, Georgoulis M, Deutsch M, Tiniakos D, et al. Adherence to the Mediterranean diet is associated with the severity of non-alcoholic fatty liver disease. Clin Nutr 2014;33:678–83. doi:10.1016/j.clnu.2013.08.014.

604 [29] Ryan MC, Itsiopoulos C, Thodis T, Ward G, Trost N, Hofferberth S, et al. The Mediterranean diet improves hepatic steatosis and insulin sensitivity in individuals with non-alcoholic fatty liver disease. J Hepatol 2013;59:138–43. doi:10.1016/j.jhep.2013.02.012.

608 [30] Hur W, Kim SW, Lee YK, Choi JE, Hong SW, Song MJ, Bae SH, Park T, Um SJ YS. Oleuropein reduces free fatty acid-induced lipogenesis via lowered extracellular signal-regulated kinase activation in hepatocytes. Nutro Res 2012;32:778–86. doi:10.1016/j.nutres.2012.06.017.

612 [31] Barbaro B, Toietta G, Maggio R, Arciello M, Tarocchi M, Galli A, et al. Effects of the olive-derived polyphenol oleuropein on human health. Int J Mol Sci 2014;15:18508–24. doi:10.3390/ijms151018508.

615 [32] Park S, Choi Y, Um S-J, Yoon SK, Park T. Oleuropein attenuates hepatic steatosis induced by high-fat diet in mice. J Hepatol 2011;54:984–93. doi:10.1016/j.jhep.2010.08.019.

618 [33] Goya L, Mateos R, Bravo L. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. Eur J Nutr 2007;46:70–8. doi:10.1007/s00394-006-0633-8.

621 [34] Hoek-van den Hil EF, van Schothorst EM, van der Stelt I, Swarts HJM, van Vliet M, Amolo T, et al. Direct comparison of metabolic health effects of the flavonoids quercetin, hesperetin, epicatechin, apigenin and anthocyanins in high-fat-diet-fed mice. Genes Nutr 2015;10:469. doi:10.1007/s12263-015-0469-z.

625 [35] Ragab SMM, Abd Elghaffar SK, El-Metwally TH, Badr G, Mahmoud MH, Omar HM. Effect of a high fat, high sucrose diet on the promotion of non-alcoholic fatty liver disease in male rats: the ameliorative role of three natural compounds. Lipids Health Dis 2015;14:83. doi:10.1186/s12944-015-0087-1.

629 [36] Liao C-C, Ou T-T, Huang H-P, Wang C-J. The inhibition of oleic acid induced hepatic lipogenesis and the promotion of lipolysis by caffeic acid via up-regulation of AMP-activated kinase. J Sci Food Agric 2014;94:1154–62. doi:10.1002/jsfa.6386.

633 [37] Grasselli E, Voci A, Canesi L, Goglia F, Ravera S, Panfoli I, et al. Non-receptor-mediated actions are responsible for the lipid-lowering effects of iodothyronines in FaO rat hepatoma cells. J Endocrinol 2011;210:59–69. doi:10.1530/JOE-11-0074.

636 [38] Grasselli E, Cortese K, Fabbri R, Smerilli A, Vergani L, Voci A, Gallo G, Canesi L.
637 Thyromimetic actions of tetrabromobisphenol A (TBBPA) in steatotic FaO rat
638 hepatoma cells. Chemosphere 2014; 112:511-8.

639 [39] Zhou H, Liu X, Liu L, Yang Z, Zhang S, Tang M, et al. Oxidative stress and
640 apoptosis of human brain microvascular endothelial cells induced by free fatty acids.
641 J Int Med Res n.d.;37:1897–903.

642 [40] Szmitko PE, Wang C-H, Weisel RD, Jeffries GA, Anderson TJ, Verma S.
643 Biomarkers of vascular disease linking inflammation to endothelial activation: Part
644 II. Circulation 2003;108:2041–8. doi:10.1161/01.CIR.0000089093.75585.98.

645 [41] Aliakbarian B, Casazza AA, Perego P. Valorization of olive oil solid waste using
646 high pressure-high temperature reactor. Food Chem 2011;128:704–10.
647 doi:10.1016/j.foodchem.2011.03.092.

648 [42] Paini M, Casazza AA, Aliakbarian B, Perego P BACG. Influence of ethanol/water
649 ratio in ultrasound and high-pressure/high-temperature phenolic compound
650 extraction from agri-food waste. Int J Food Sci Tech 2016;51:349–58.

651 [43] Vergani L, Vecchione G, Baldini F, Voci A, Ferrari PF, Aliakbarian B, Casazza AA
652 PP. Antioxidant and hepatoprotective potentials of phenolic compounds from olive
653 pomace. Chem Eng Trans, 2016, p. 475–80.

654 [44] Gutfinger T. Polyphenols in olive oils. J Am Oil Chem Soc 1981;58:966–8.
655 doi:10.1007/BF02659771.

656 [45] Lauris V, Crettaz M, Kahn CR. Coordinate roles of insulin and glucose on the
657 growth of hepatoma cells in culture. Endocrinology 1986;118:2519–24.
658 doi:10.1210/endo-118-6-2519.

659 [46] Wiechelman KJ, Braun RD, Fitzpatrick JD. Investigation of the bicinchoninic acid
660 protein assay: identification of the groups responsible for color formation. Anal
661 Biochem 1988;175:231–7.

662 [47] Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo
663 and in cell culture: how should you do it and what do the results mean? Br J
664 Pharmacol 2004;142:231–55. doi:10.1038/sj.bjp.0705776.

665 [48] Iguchi H, Kojo S, Ikeda M. Lipid peroxidation and disintegration of the cell
666 membrane structure in cultures of rat lung fibroblasts treated with asbestos. J Appl
667 Toxicol 13:269–75.

668 [49] Grasselli E, Voci A, Pesce C, Canesi L, Fugassa E, Gallo G, et al. PAT protein
669 mRNA expression in primary rat hepatocytes: Effects of exposure to fatty acids. Int
670 J Mol Med 2010;25:505–12.

671 [50] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS TS. Analysis of
672 nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–8.

673 [51] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-

674 PCR. Nucleic Acids Res 2001;29:e45.

675 [52] Grasselli E, Compalati AD, Voci A, Vecchione G, Ragazzoni M, Gallo G, Borro P,
676 Sumberaz A, Testino G, Vergani L. Altered oxidative stress/antioxidant status in
677 blood of alcoholic subjects is associated with alcoholic liver disease. Drug Alcohol
678 Depend 2014;143:112-9.

679 [53] Benvenuto F, Voci A, Carminati E, Gualandi F, Mancardi G, Uccelli A, Vergani L.
680 Human mesenchymal stem cells target adhesion molecules and receptors involved in
681 T cell extravasation. Stem Cell Res Ther 2015;6:245.

682 [54] Laemmli UK. Cleavage of structural proteins during the assembly of the head of
683 bacteriophage T4. Nature 1970;227:680–5.

684 [55] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from
685 polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc
686 Natl Acad Sci U S A 1979;76:4350–4.

687 [56] Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong C-P, Nettleton JA, et al.
688 Flavonoid intake and cardiovascular disease mortality: a prospective study in
689 postmenopausal women. Am J Clin Nutr 2007;85:895–909. doi:85/3/895 [pii].

690 [57] Covas M-I, Nyyssönen K, Poulsen HE, Kaikkonen J, Zunft H-JF, Kiesewetter H, et
691 al. The effect of polyphenols in olive oil on heart disease risk factors: a randomized
692 trial. Ann Intern Med 2006;145:333–41. doi:145/5/333 [pii].

693 [58] Hooper L, Kay C, Abdelhamid A, Kroon P a, Cohn JS, Rimm EB, et al. Effects of
694 chocolate , cocoa , and flavan-3-ols on cardiovascular health : a systematic review
695 and meta-analysis of randomized trials 1 – 3. Am J Clin Nutr 2012;95:740–51.
696 doi:10.3945/ajcn.111.023457.INTRODUCTION.

697 [59] Bower A, Marquez S, De Mejia EG. The Health Benefits of Selected Culinary
698 Herbs and Spices Found in the Traditional Mediterranean Diet. Crit Rev Food Sci
699 Nutr 2015. doi:10.1080/10408398.2013.805713.

700 [60] Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte
701 inflammatory cytokines. Nature 1998;391:82–6. doi:10.1038/34184.

702 [61] Byrne CD. Fatty liver: role of inflammation and fatty acid nutrition. Prostaglandins
703 Leukot Essent Fatty Acids 82:265–71. doi:10.1016/j.plefa.2010.02.012.

704 [62] Lalor PF, Shields P, Grant A, Adams DH. Recruitment of lymphocytes to the human
705 liver. Immunol Cell Biol 2002;80:52–64. doi:10.1046/j.1440-1711.2002.01062.x.

706 [63] Roebuck KA, Finnegan A. Regulation of intercellular adhesion molecule-1 (CD54)
707 gene expression. J Leukoc Biol 1999;66:876–88.

708 [64] Kuhlencordt PJ, Rosel E, Gerszten RE, Morales-Ruiz M, Dombkowski D, Atkinson
709 WJ, et al. Role of endothelial nitric oxide synthase in endothelial activation: insights
710 from eNOS knockout endothelial cells. Am J Physiol Cell Physiol 2004;286:C1195–
711 202. doi:10.1152/ajpcell.00546.2002.

712 [65] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and
713 disease. Physiol Rev 2007;87:315–424. doi:10.1152/physrev.00029.2006.

714

Figure1

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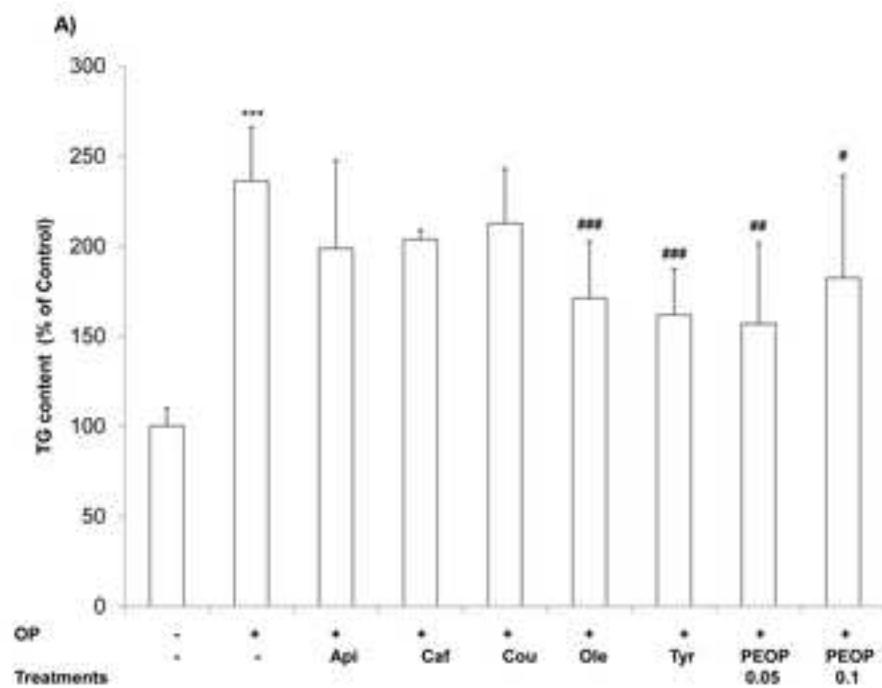

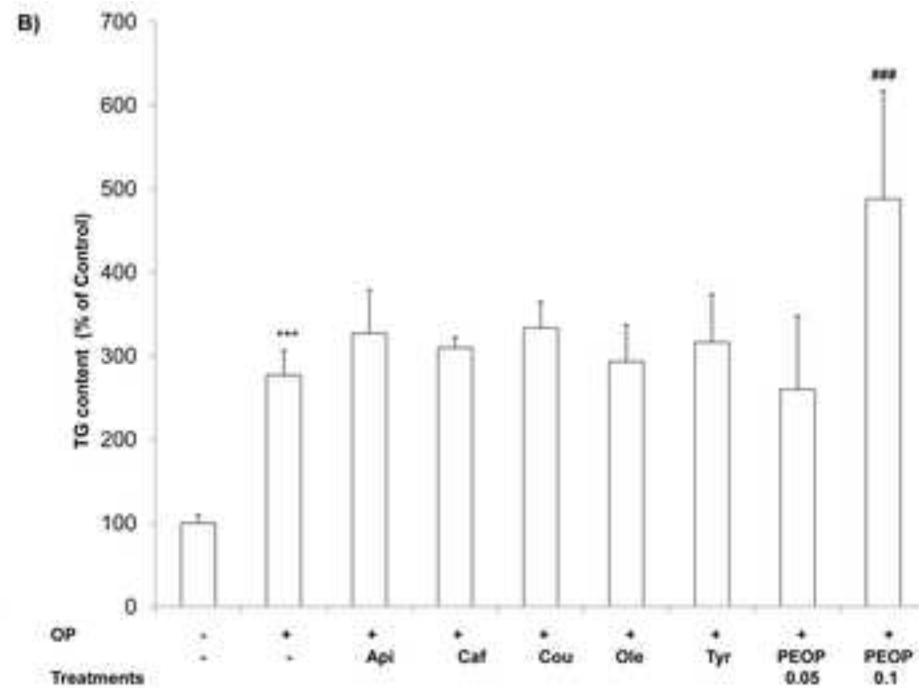

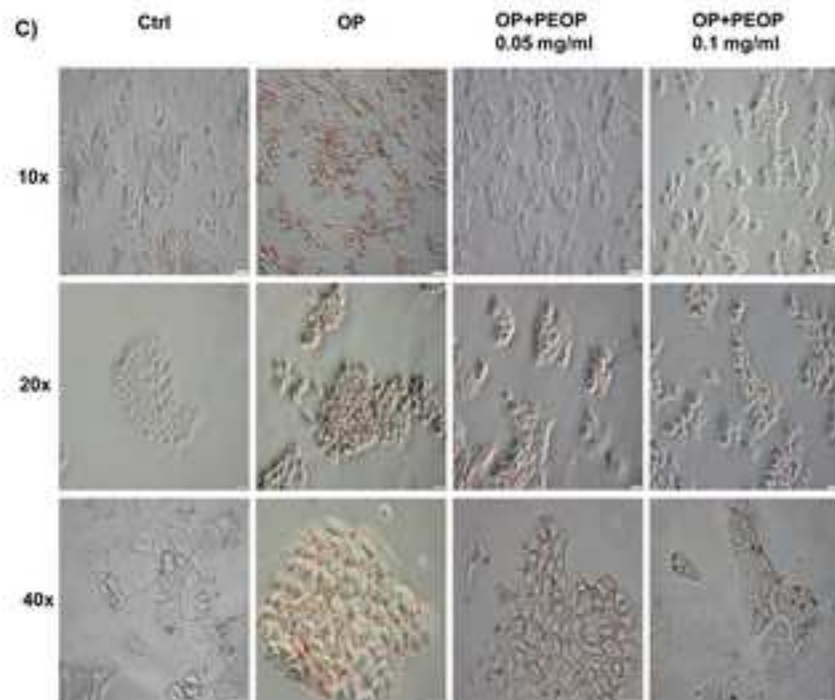

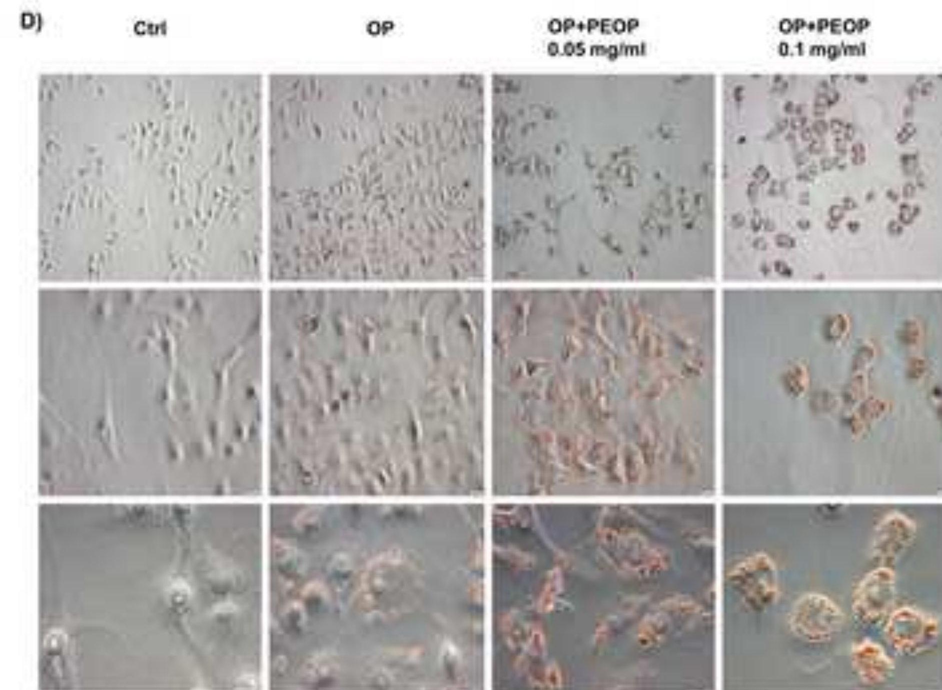

Figure2

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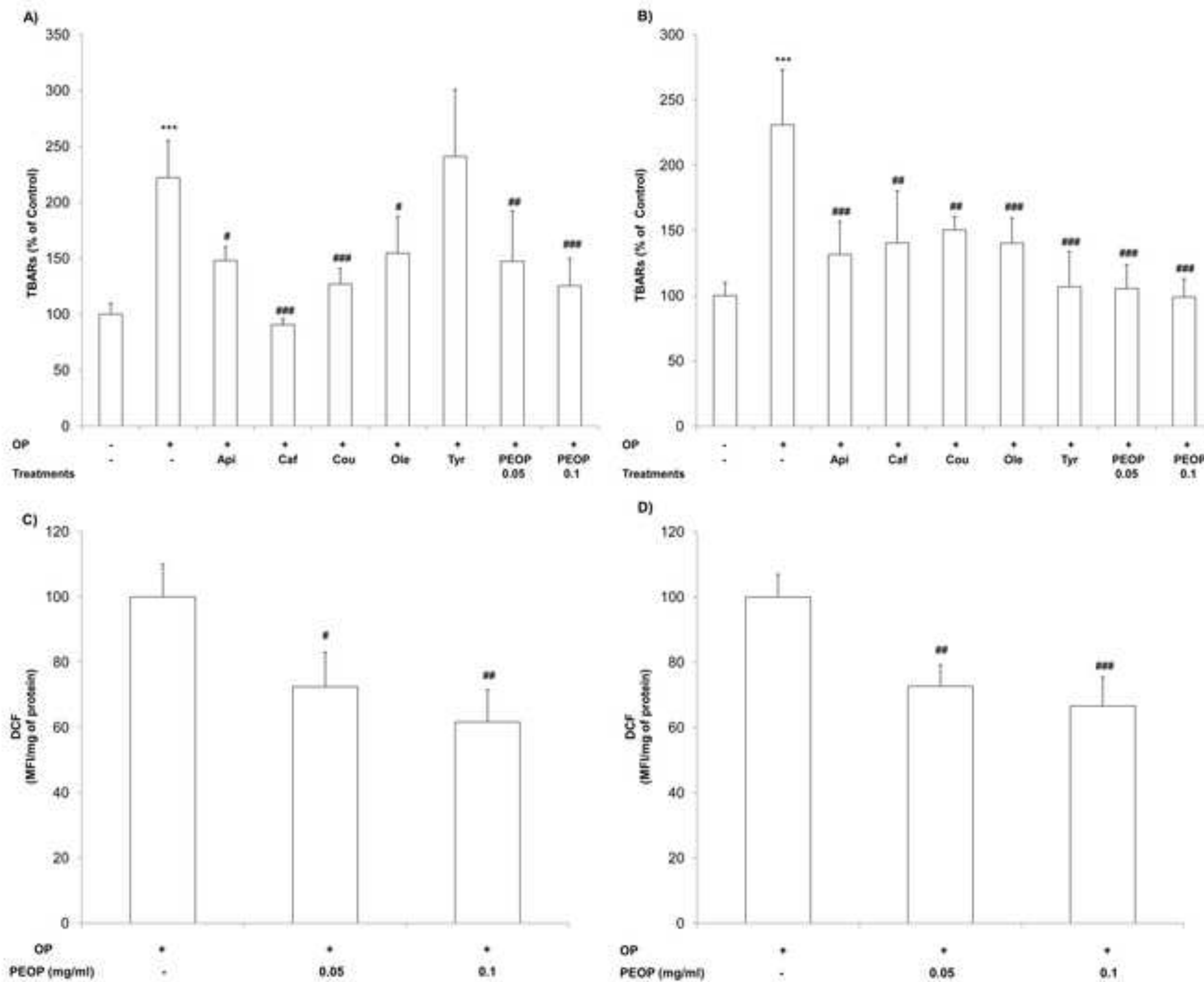

Figure3

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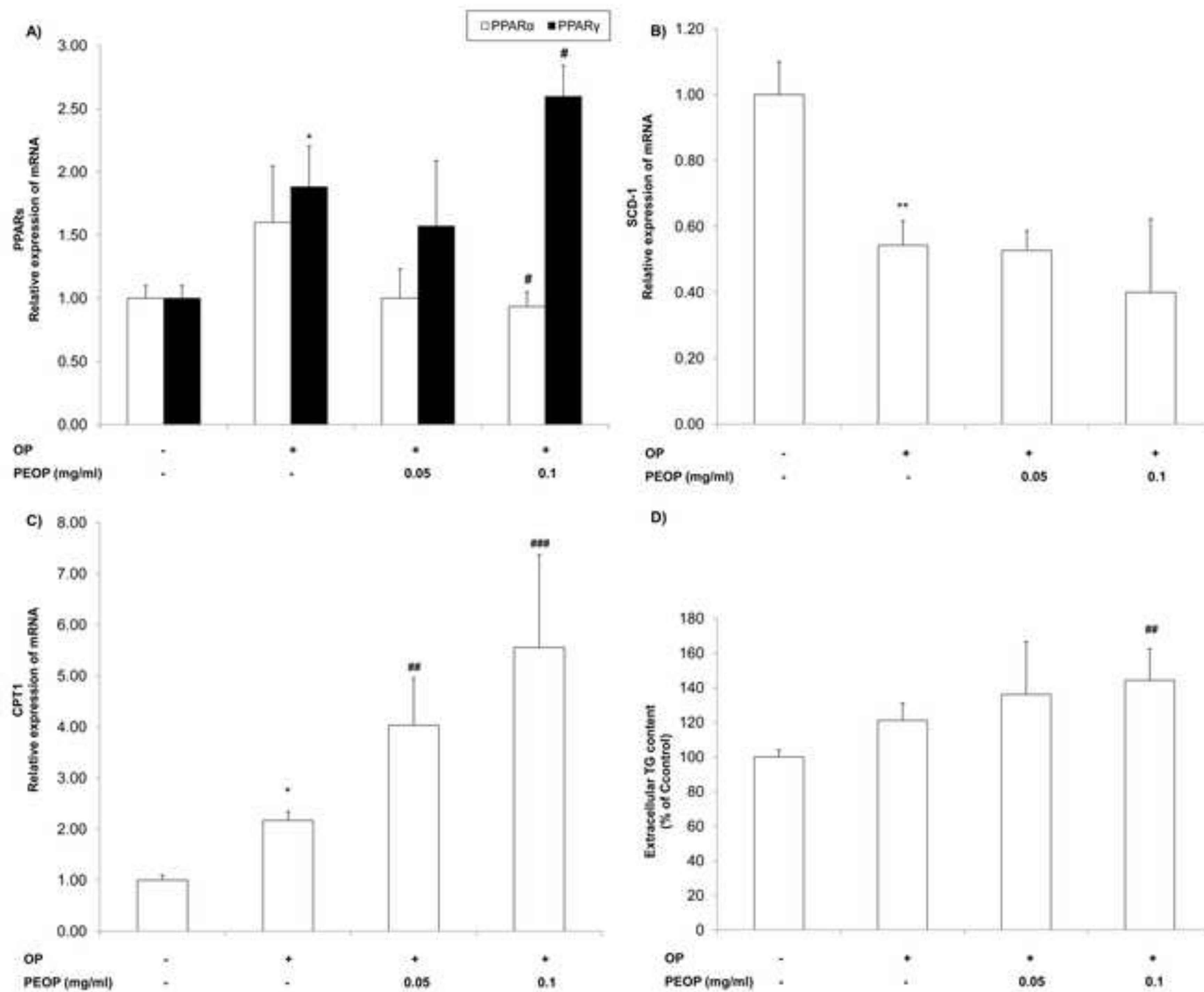
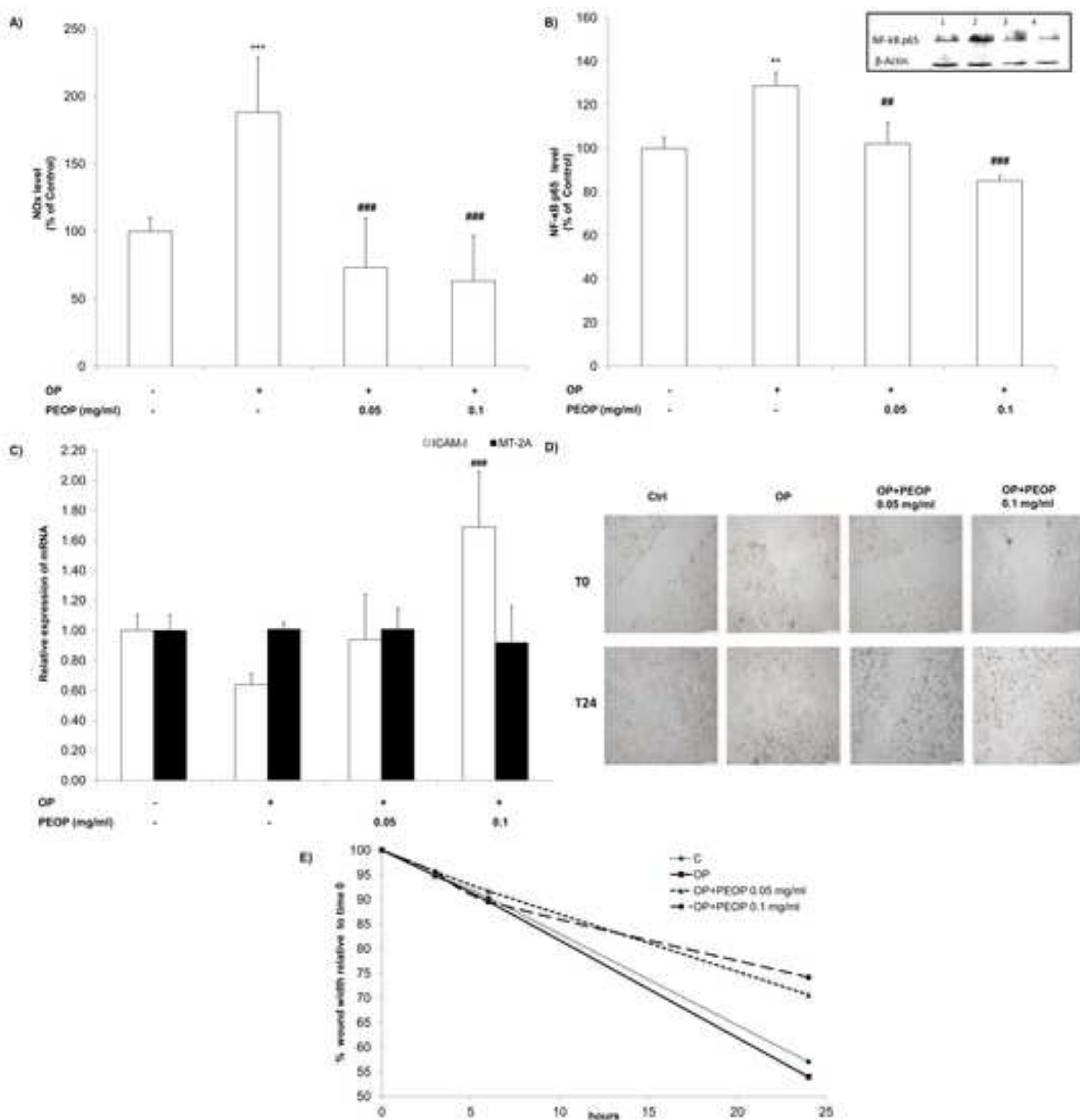


Figure4

Click here to download Figure FIG4.TIF.tif



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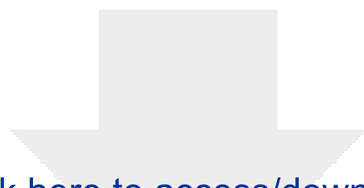
Phenolic compound	**Concentration (mg/mL)**
Tyrosol	0.10
Caffeic acid	0.03
Coumaric acid	0.03
Oleuropein	0.49
Apigenin	0.01

Graphical abstract



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